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## IMMUNOCHEMISTRY OF RAT LUNG TUMORIGENESIS

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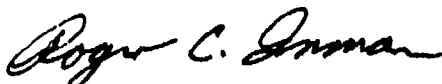
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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

**FOR THE COMMANDER**



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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Changes in cell mediated immunity have been identified in relation to tumorigenesis. Most chemical carcinogens were found to be immunosuppressive. Cytogenetic changes and impaired DNA repair-synthesis were also found to be associated in chemical carcinogenesis. Measurement of temporal variability of immunologic, cytogenetic and DNA repair changes in relation to exposure to chemical carcinogens and throughout initiation and progression of lung cancer in an animal model is the subject of this research project. The first phase of		

this investigation included the in vitro measurement of cellular immune response as well as sister chromatid exchange and DNA replication and repair synthesis in spleen, thymus and blood lymphocytes in control rats. Phase II included the measurement of cellular immune parameters, sister chromatid exchange and DNA replication and repair synthesis in spleen, thymus and blood lymphocytes in early tumorigenesis in rats intratracheally exposed to 3-methylcholanthrene.

## PREFACE

This is the second Annual Report for the Project on Immunochemistry of Rat Lung Tumorigenesis, a subprogram of the Toxic Hazards Research program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under Contract Number AF F33615-80-C-0512. This report describes the research activities at UCI from June 1981 through August 1982. During this period, H.A. Guirgis, Ph.D. was Principal Investigator for the research project.

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## INTRODUCTION

The study of specific tumor antigens of chemically induced neoplasms was continued by the demonstration that neoantigens of malignant cells provided the immunizing stimulus and that resistance to tumor reimplantation could be developed in the autochthonous host (Prehn, 1957; Klein et al., 1960). A large number of chemically induced tumors were shown to be antigenic (Baldwin, 1967; Prehn, 1962). Even tumors induced by physical means such as ultraviolet radiation possess neoantigens although their antigenicity is weak (Klein et al., 1963). Chemically induced tumors possess individual specificity. This display of individual specificity might support the view that these antigens are expressions of various gene groups of the host cell. However, cross-reactivity does occur in chemically induced tumors.

The relationship of locally growing tumors to their host is variable. These host-tumor relationships reflect the interactions of many factors (Vaage, 1974; Mikulska, 1966): the need for continuous stimulation of the host by tumor antigens for the maintenance of immunity; immunosuppressive effects of large tumors mediated possibly by release of excessive amounts of circulating soluble tumor antigens that preempt potentially cytotoxic lymphocytes; development of suppressor lymphoid cell populations; and blocking serum factors (Riggins, 1964; Stjernsward, 1968; Lausch, 1969; Bard, 1969; Chandradasa, 1973; Basombrio, 1972; Baldwin, 1974; Yoshida, 1963; Rosenau, 1966). However, spleen, lymph node or peritoneal cells of tumor-bearing animals are often capable of expressing in vitro and transferring in vivo anti-tumor immunity (Deckers, 1971; Wepsic, 1970; Milas, 1974). The magnitude of this activity depends on the tumor growth rate and size of the tumor.

Suppressor function of immune responsiveness has been attributed to a special class of T lymphocytes. The suppressor cells, which normally maintain homeostasis by preventing auto-immune reactions, do so either by suppressing helper and amplifying T cells or by suppressing specifically the activity of non-T cells; antibody production by B cells is not commonly the subject of suppressor T cell activity. Suppressor T cells exert their effect on the tumor-bearing host by molecular mediators with a molecular weight lower than that of the serum albumin (Fujimoto, 1975). The targets of regulatory activity of suppressor T cells are both B and T lymphoid cells. Interactions between T cells are thought to be well balanced; i.e., for lung T cell dependent augmentation (helper and amplifier function) there is an equal but opposite T cell mediated

suppression (Gershon, 1974). Antigen-antibody complexes and certain mitogens such as Concanavalin-A induce suppressor cells (Glasgow, 1974; Rich, 1975). Suppressor T cells could be determined using colony inhibition assay, microplate cytotoxicity assays, or using mediators such as cimetidine or Indomethacin. In mice with tumors exceeding a certain size, rapid decrease and disappearance of all cytotoxic activity followed. Three or four weeks after surgical removal of tumor, the cytotoxicity was fully restored. This fluctuation of lymphocytic cytotoxicity depended on tumor size (Youn, 1973; LaFrancois, 1974).

In 1959 it was reported that phytohemagglutinin (PHA), an extract of the red kidney bean (*Phaeolus Vulgaris*), could stimulate the transformation of human small lymphocytes in culture (Hungerford, 1959). The in vitro lymphocyte response to PHA was found to be of value in the classification of the primary immunologic deficiency diseases (Seligmann, 1968). Work in experimental animals suggested that PHA induces the transformation of T lymphocytes (Rodey, 1969; Greaves, 1968; Doenhoff, 1970; Owen, 1971; Jones, 1972; Lischner, 1967). Recent findings indicate that at least a portion of those lymphocytes responding to PHA are also B lymphocytes (Philips, 1973). PHA was the first nonspecific stimulant of lymphocyte transformation discovered (Oppenheim, 1968). A number of other stimulants of in vitro lymphocyte transformation have also been found. These included Pokeweed mitogen (PWM), an extract of *Phytolacca Americana*, and Concanavalin A (Con A). PWM appears to stimulate mainly B lymphocytes (Knudsen, 1974; Weber, 1973; Weksler, 1974; Cisco, 1974; Stockman, 1971), Con A stimulates T lymphocytes (Jondal, 1975); these may represent T lymphocytes at different stages of maturation than those stimulated by PHA (Stobo, 1973). Con A will also stimulate B lymphocytes (Chess, 1974). The number of T lymphocytes in peripheral blood has a direct positive correlation with the in vitro lymphocyte response to PHA and a direct negative correlation with the in vitro lymphocyte response to PWM (Sengar, 1975).

Impairment of that response has been found in lymphocytes from animals with tumors and from non-tumor-bearing animals after exposure to chemical carcinogens (Mekler et al., 1974). The cellular immune response may also be impaired in different disease processes. This has been found in both animal and human studies (Ben-Bassat et al., 1975; Ambrogi et al., 1976; Schumm et al., 1974; Liao et al., 1972). The magnitude of the impairment of lymphocyte response to mitogens has been correlated with the localization of the tumor and the tumor burden. Transformation is quantitated microscopically (blast-forming cells) or by measuring the rate of

incorporation of  $^3\text{H}$ -thymidine following culturing of the cells in the presence of mitogens. In the present study we have used PHA, Con A, and LA on spleen and thymus lymphocytes. These mitogens are polyclonal activators because they react with the cell surface nonspecifically to produce the same series of cellular events.

Sister chromatid exchange (SCE) has been studied in animal models, both in vivo and in vitro, as well as in human cells such as skin fibroblasts and lymphocytes. Data suggest that SCE might be useful in testing carcinogenicity, mutagenicity and predisposition to certain diseases.

Sister chromatid exchanges were first identified by Taylor (1958) in metaphase cells that had replicated in the presence of  $^3\text{H}$ -thymidine. SCEs are demonstrated as indicators of DNA damage which might be induced by a variety of chemical or physical agents. SCE has been studied in cells from different species including Chinese hamster cells (Perry and Wolff, 1974), human cells (Latt, 1974; Wolff et al., 1975), mouse fetal liver cells (Alvarez et al., 1980), and others (Galloway et al., 1980; Kram, 1980; Carrano and Thompson, 1982). The level of SCE's has been shown to increase following in vivo and in vitro exposures to agents which damage DNA. These include physical agents such as ultraviolet light, and chemical agents such as alkylating agents, polycyclic aromatic hydrocarbons, aflatoxin B<sub>1</sub> and others (Wolff, 1977; Perry, 1980; Carrano et al., 1978). Within a species there may be wide intra- and inter-individual variation in SCE frequency. The variation between individuals in humans does not seem to depend on age or sex (Schneider, 1977; Lambert, 1978); however, smokers show a higher SCE rate than nonsmokers (Lambert, 1978). It has been suggested that differences in genotype may explain differing SCE frequencies between individuals of a given species (Galloway and Evans, 1975). Although the exact mechanism of SCE formation remains unknown, it was postulated that it may be due to mutagenic lesions (Carrano and Moore, 1981; Carrano and Thompson, 1981).

Some agents, such as UV light and ionizing radiation, that stimulate DNA repair also stimulate SCE formation (Bender et al., 1974); hence, a number of investigators have concluded that SCE's are related to repair of damaged DNA and, more specifically, to postreplication repair (Beck and Obe, 1975; Galloway, 1977; Latt and Juergens, 1977). A recent study demonstrated a correlation between induction of mutation at a single gene locus, the gene for the enzyme hypoxanthine phosphoribosyl transferase (HPRT), and of SCEs (Carrano et al., 1978). This correlation and evidence that the same agents induce SCEs at low concentrations and chromosome aberrations

at higher concentrations have led some investigators to propose SCE studies to determine the mutagenic and carcinogenic potential of those chemicals (Beck and Obe, 1975; Carrano et al., 1978).

### PURPOSE

The purpose of this research project was to quantitate temporal changes in cellular immune measurements, sister chromatid exchange and unscheduled DNA synthesis in lymphocytes from spleen, thymus and blood of rats exposed intratracheally to 3-methylcholanthrene (MCA).

### SPECIFIC AIMS

The specific aims of this project were to longitudinally quantitate the parameters given below in lymphocytes from spleen, thymus and blood of control rats and in rats after short term intratracheal exposure to MCA. Measurements were also done on rats intratracheally exposed to several doses of MCA to look for dose-response relationship, and to rats exposed to repeated carcinogenic doses of MCA.

#### Measurements of cellular immune response

- a. Lymphocyte stimulation using mitogens Phytohemagglutinin (PHA), Leucoagglutin (LA) and Concanavalin A (Con A).
- b. Quantitation of function of lymphocyte suppressor cell subpopulation.

Quantitation of sister chromatid exchange.

DNA replication and repair synthesis.

### METHODS AND PROCEDURES

#### ANIMAL MODEL

The Fischer 344 rat was selected for this study. In this animal model, broncho-alveolar squamous lung tumors could be induced using intratracheal inoculations of 3-methylcholanthrene (MCA).

The first attempt to induce lung tumors in Fischer 344 rats by the intratracheal (i.t.) inoculation of a suspension of 3-methylcholanthrene (MCA) in gel-saline was not successful (this work was done at the THRU). The negative result was attributed to the small particle size of the MCA (ca. 1  $\mu$ m). A second attempt was

initiated in late January 1982 using a preparation of MCA having particles in the range of 100  $\mu$ m. This procedure was adopted after consultation with other investigators who have induced tumors in mice with MCA suspensions. The protocols and dosages are essentially the same as for the first attempt, except for the MCA particle size. The first tumors were formed in April 1982 (10 weeks after first treatment).

## LABORATORY METHODS

The following are brief descriptions of laboratory methods to measure cellular immune response. Methods to determine sister chromatid exchanges, DNA repair and DNA replication in lymphocytes will also be described.

### Separation of Spleen Lymphocytes and Thymocytes

#### Method.

The organs (spleen and thymus) are removed intact under aseptic conditions and placed in sterile tubes containing Roswell Park Memorial Institute 1640 (RPMI) tissue culture medium. The extra tissue surrounding the organs is dissected free and discarded; this procedure is done in a petri dish using RPMI to keep the tissue moist.

The cleaned spleen is then transferred to a second petri dish containing RPMI where it is cut into 3 pieces and gently mashed using a tissue squasher. The cell suspension is collected into a tube leaving the large chunks in the petri dish. More RPMI is added and the chunks are resquashed. This suspension is collected and added to the same tube. The thymus is treated similarly to spleen but cell suspension is prepared using a homogenizer.

The cell suspensions may be pooled from 2-4 animals if treatments are the same. The cells are washed 3 times before counting using a coulter counter. After the first wash the suspensions are allowed to sit for several minutes to allow large chunks to settle to the bottom. The supernatant is aspirated and spun and washed 2 more times.

## Mitogen Stimulation and Function of Suppressor T Cell

### Background.

In vitro response of lymphocytes to mitogens, particularly phytohemagglutinin (PHA), has been widely used to demonstrate a defect in cellular immune mechanisms. Mekori (1974) demonstrated that lymphocytes from patients with generalized malignant disease were less responsive to PHA stimulation than lymphocytes from patients with localized disease. Other authors showed a correlation between stage of disease and immune impairment using PHA and Con A lymphocyte stimulation from patients with bronchogenic carcinoma (Liebler et al., 1977).

Lymphocyte-reactivity to mitogens was also studied in animal models to test the presence of an association with malignant disease and with aging (Barker and Moore, 1977; Metcalf and Moulds, 1967; Nagaya, 1973). A decrease in the reactivity of spleen lymphocytes to PHA with age and disease onset has been reported (Metcalf and Moulds, 1967).

Lymphocytes suspended in RPMI supplemented with 20% fetal calf serum, L-glutamine and gentamicin are used to test mitogen activation. The mitogens used are Concanavalin A (Con A), Leucoagglutinin (Leuco) and phytohemagglutinin (PHA) at 3 different concentrations. Cimetidine (CIM) and Indomethacin (INDO) are used to test suppressor T cell activity. All mitogen-containing cultures are prepared in quadruplicate in microculture plates and incubated for 72 hours at 37°C in a 5% CO<sub>2</sub> humidified incubator. The cultures are then labeled with 12.5 µCi/ml of <sup>3</sup>H-thymidine during the final 6 hours of incubation. The samples are harvested on a multiple automatic sample harvester (MASH) using glass fiber filter strips which are then dried and subsequently placed in scintillation fluid for counting in a liquid scintillation spectrometer.

## DNA Replication and Repair Synthesis in Lymphocytes

### Background.

DNA repair synthesis has been studied in vitro in humans and in vivo and in vitro in animal models after treatment with carcinogens as well as with chemotherapeutic agents. This is usually done by measuring the incorporation of <sup>3</sup>H-thymidine into non-replicating DNA (Stich and Keiser, 1974; Smith and Hanawalt, 1976a,b; Cleaver, 1973; Craddock et al., 1976; and Lieberman et al., 1971). The increased incorporation of <sup>3</sup>H-thymidine in non-replicative cells is termed

"repair" or "unscheduled DNA synthesis." This is a subject of controversy (Melzer, 1979). However, it seems that this phenomenon, if not indicative of DNA repair, suggests a direct carcinogen-DNA interaction or carcinogen-induced replication DNA synthesis.

#### Method.

#### Preliminary preparation of equipment and cells:

Lymphocytes are separated according to the method described previously. Cells are suspended in each of 2 media: (1) RPMI 20% fetal calf serum (FCS), Fluorodeoxyuridine (FU) or (2) RPMI, FCS, FU and Hydroxyurea (HU). Cultures are set up in quintuplicate and 12 wells of background media are set up. Cells are plated in flat bottom microtiter plates and exposed to UV light for 20 seconds at a dose of 10 microamperes. Control cells are covered with tin foil to block UV light.  $^3\text{H}$ -Thymidine is added to the cultures giving a final concentration of 1  $\mu\text{C}$ /well. Cultures are incubated for 3 hours at 37°C in humid  $\text{CO}_2$  (5%) incubator. Samples are harvested on a multiple automatic sample harvester (MASH) using glass fiber filter strips which are then dried and placed in ASC scintillation fluid for counting in a TRACOR scintillation counter. Background counts are averaged and subtracted from the control and UV cell counts.

#### Sister Chromatid Exchange

Lymphocytes (methods described above) are cultured at a concentration of  $0.5 \times 10^6$  cells/ml in a total volume of 3 ml/culture using the following medium: Roswell Park Memorial Institute 1640 (RPMI 1640) (Grand Island Biological Co.), 1 mM/ml Glutamine (Grand Island Biological Co.), 100  $\mu\text{g}$ /ml Gentamicin (Upjohn), 20% fetal calf serum (Reheis), 4% Phytohemagglutinin, (PHA), (Grand Island Biological Co.), 1.67% bromodeoxyuridine (BUdR). The cultures are incubated for 72 hours at 37°C in a 5%  $\text{CO}_2$  humidified incubator. At 68 hours Colcemid (Grand Island Biological Co.) is added to the cultures to give a final concentration of 0.05  $\mu\text{g}$  colcemid per ml of culture medium. The cultures are returned to a  $\text{CO}_2$  incubator for a period of 4 hours after which they are treated with a solution of 0.075 M KCl, fixed with a 3:1 mixture of methanol and acetic acid, and chromosome spreads are prepared on microscope slides. The slides are air-dried and then stained with Hoechst 33258 at a concentration of 50  $\mu\text{g}$ /ml in Sorensen's buffer for 10 minutes and rinsed in distilled  $\text{H}_2\text{O}$ . Slides are placed in a shallow pan, flooded with Sorensen's buffer, covered with saran wrap and exposed to intense illumination from cool-white fluorescent



lamps for 3 hours. They are stained with a solution of 3% Gurr's R-66 Giemsa (Searle) in sodium phosphate buffer for 15 minutes, rinsed in sodium phosphate buffer (pH 6.8), and left to air dry overnight before cover slipping.

## RESULTS

The results will be presented first on spleen and thymus lymphocytes from control rats followed by results on lymphocytes from rats intratracheally exposed to 3-methylcholanthrene.

### IMMUNOCHEMISTRY STUDIES IN CONTROL RATS

#### In Vitro Mitogen Stimulation-Spleen and Thymus Lymphocytes from Control and MCA Treated Animals

In vitro response of lymphocytes to specific antigens and plant mitogens has been used to demonstrate the level of cellular immune response. Impairment of the response upon in vitro testing has been found to be associated with the presence of malignant disease in both animal models and in human experience. Furthermore, the magnitude of the impairment in lymphocyte response to mitogens has been correlated with tumor burden.

To develop the methods for mitogen stimulation the factors tested included different mitogens and several doses are used. The results of the effects of those factors are included in Figures 1, 2, and 3. Dose response relationships of PHA mitogen stimulation in rat spleen lymphocytes in groups of control animals and rats intratracheally exposed to 3-MCA are examined. The effect of 3-MCA treatment on mitogen stimulation using PHA, Leuco and Con A was measured. The results are presented in Tables 1, 2, and 3. As can be seen, there is a trend of immunosuppression after exposure to MCA.

#### Suppressor T Lymphocyte Function

The purpose of the following experiment was to quantitate suppressor T cell function using Cimetidine (CIM) and Indomethacin (INDO) in vitro in cultured spleen lymphocytes.

CIM and INDO are both pharmacologic agents which have been used in in vitro tests to test suppressor cell activity and thus may be valuable in quantitating the function of this lymphocyte subpopulation. CIM (N-cyano-N-methyl-N-[2-[[[5-methyl-1H-imidazol-4-yl)methyl]thio]-ethyl]guanidine) competitively inhibits

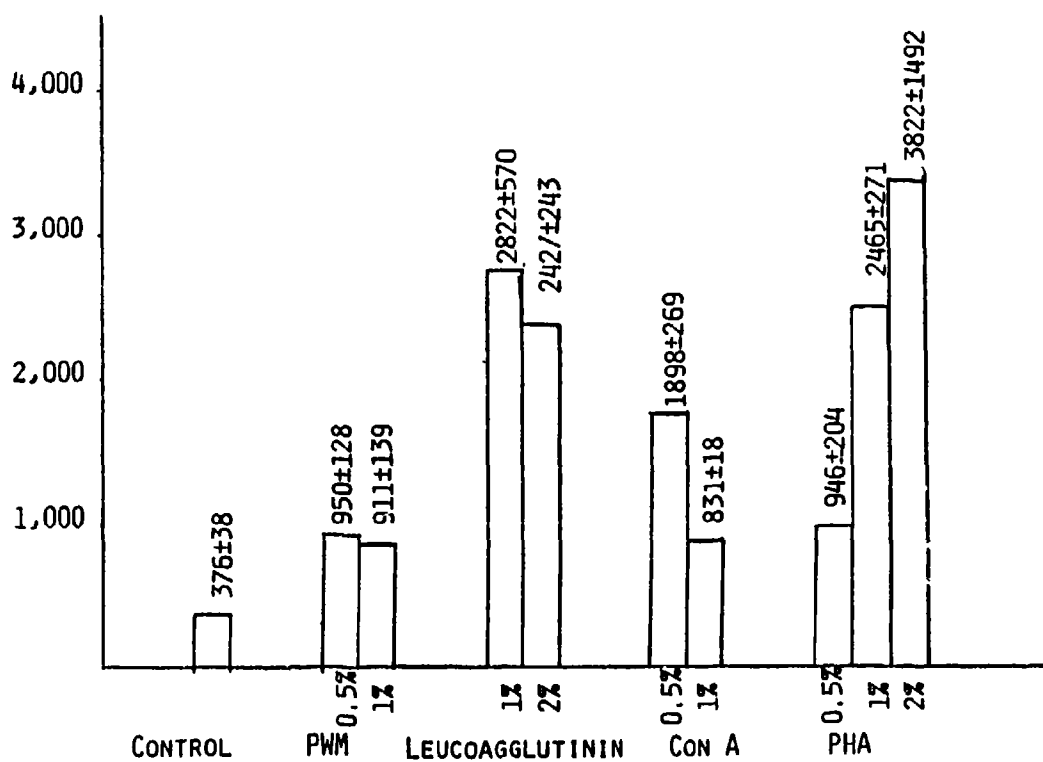


FIGURE 1

RAT SPLEEN LYMPHOCYTE STIMULATION  
USING DIFFERENT MITOGENS

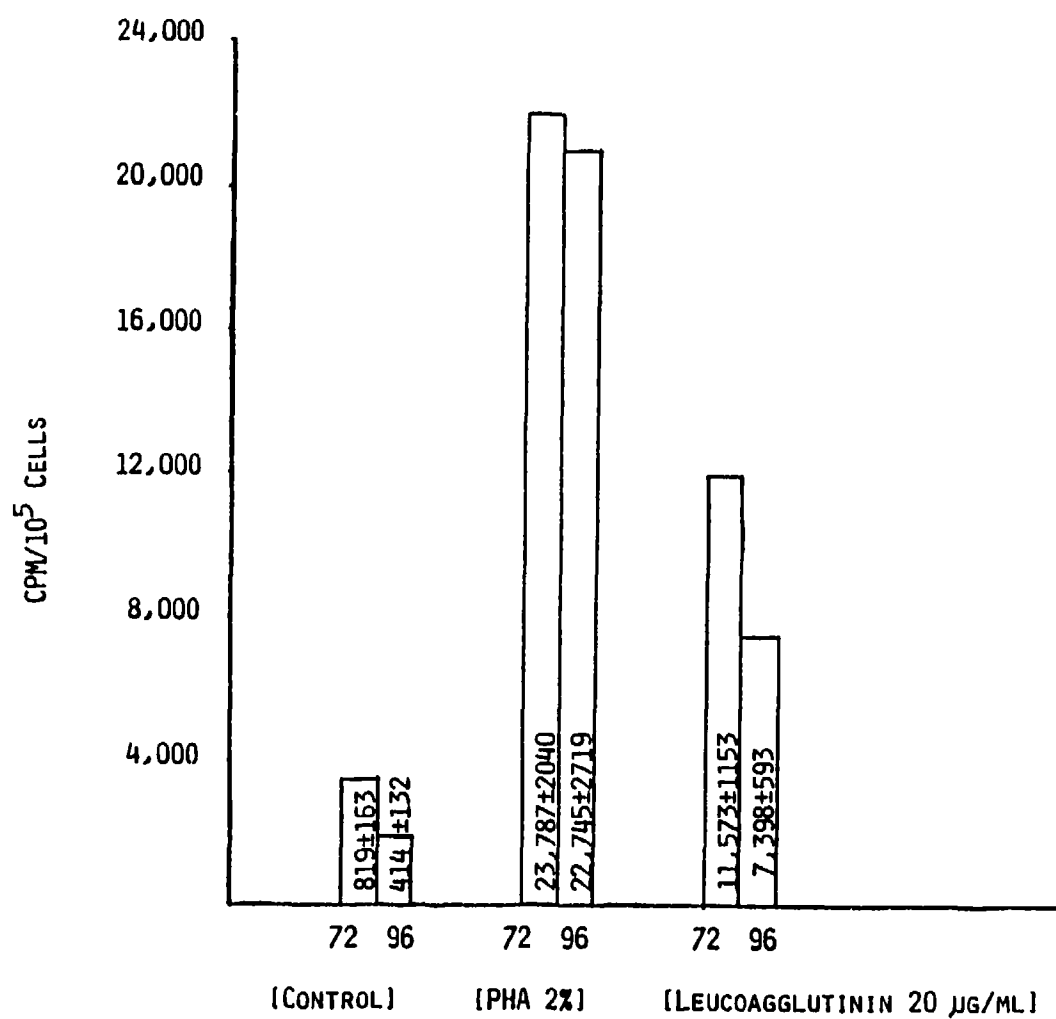


FIGURE 2 EFFECT OF CULTURE TIME ON MITOGEN ACTIVATION  
IN RAT SPLEEN LYMPHOCYTES

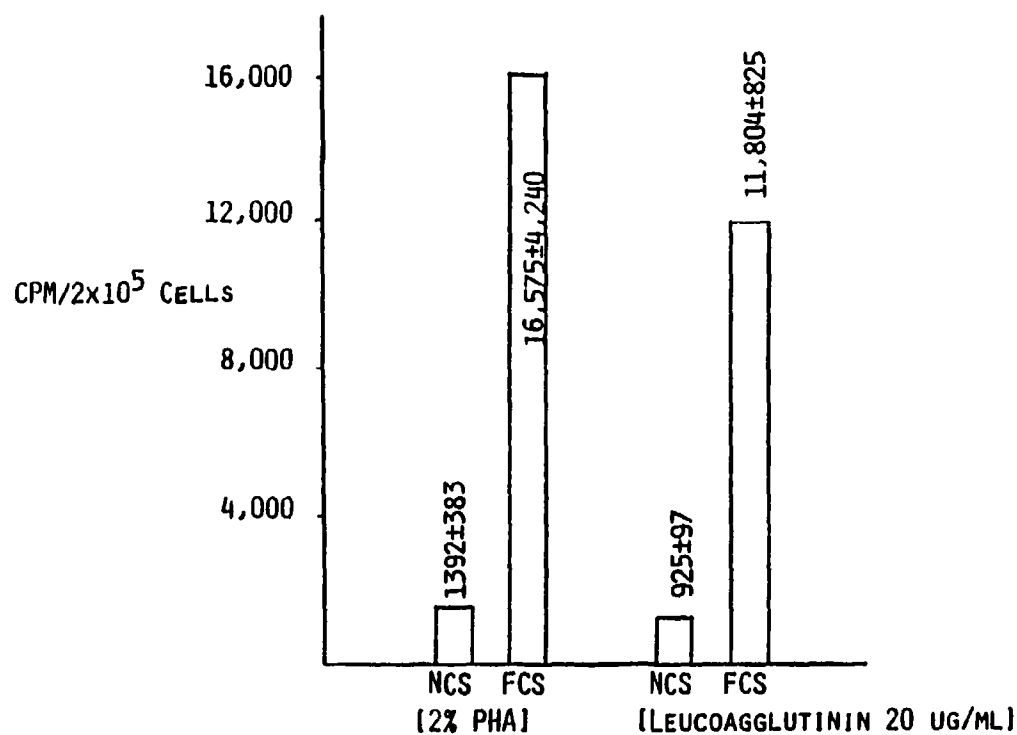


FIGURE 3

EFFECT OF DIFFERENT SUPPLEMENT ON  
MITOGEN ACTIVATION IN RAT SPLEEN LYMPHOCYTES

TABLE 1: Mitogen Activation

Phytohemagglutinin (PHA) Leucoagglutinin (Leuco) and Concanavalin-A (Con A) mitogenic activation in spleen lymphocytes of 5 groups of rats 48 hours post intratracheal treatments. Mitogenic activation is measured by <sup>3</sup>H-thymidine incorporation into the DNA of cultured lymphocytes and quantitated in disintegrations per minute (DPM).

ID	TREATMENT	CELLS WITH NO MITOGEN	PHA				LEUCO 1%	CON A 0.5%
			1%	2%	4%			
RP 79,80	Untreated	854±253	12345±1606	7587±1653	14976-2660		5673±737	66133±13079
RP 81,82	Anesthesia	774±228	14523±3666	15200±2668	32417±5579		6665±640	69209±10148
RP 83,84	Sham Surg:ry	762±153	11683±3110	12456±5152	25153±2120		7059±1347	46812±4250
RP 85,86	Ge1 Saline	946±220	13288±2160	21507±1804	29160±1483		8085±730	53316±12181
RP 87,88	3MCA	521±78					6010±1274	32844±8340

**TABLE 2:** Mitogen Activation in Rat Spleen Lymphocytes 7 and 14 Days  
After Intratracheal Instillation of 3 Methylcholanthrene (MCA)

**A. 7 Days After Treatment**

Treatment	DPM $\pm$ S.D.*			
	Control Cells	PHA 1X	PHA 2X	PHA 4X
Average of Control Rats	834 $\pm$ 85	12959 $\pm$ 1233	14187 $\pm$ 5807	25426 $\pm$ 5579
MCA treated Rats	521 $\pm$ 78	8384 $\pm$ 2158	6356 $\pm$ 1108	9142 $\pm$ 1676

**B. 14 Days After Treatment**

Treatment	DPM $\pm$ S.D.*			
	Control Cells	PHA 2X	PHA 4X	PHA 6X
Average of Control Rats	2523 $\pm$ 261	29548 $\pm$ 3271	2328 $\pm$ 3198	17757 $\pm$ 3742
MCA treated Rats	1591 $\pm$ 430	25441 $\pm$ 3938	18684 $\pm$ 2178	6886 $\pm$ 637

\*4 replicates per experiment

TABLE 3: Mitogen Activation Using ConA and Leuco in Spleen Lymphocytes  
From Control and MCA Treated Animals (DPM <sup>3</sup>H-thymidine)

DPM  $\pm$  S.D.\*

Treatment	ConA		Leuco	
	7 Days	14 Days	7 Days	14 Days
Control (Untreated)	65617 $\pm$ 4250	30489 $\pm$ 6820	6871 $\pm$ 997	18272 $\pm$ 4850
MCA	32844 $\pm$ 4692	26619 $\pm$ 4976	6010 $\pm$ 1274	6532 $\pm$ 617
% MCA of Control	50.1	87.3	87.5	35.7

\*4 Replicates Per Experiment

the action of histamine at the histamine  $H_2$ -receptors on lymphocytes. Histamine was reported to serve as a negative feedback regulator of immune response by stimulating the  $H_2$ -receptors of suppressor cells to make histamine-induced suppressor factor (HSF). In culture with T cell mitogens (such as PHA) CIM blocks the inhibitory effects of histamine in suppressor cells. Therefore, an increase in DNA synthetic activity of cells after CIM treatment indicates suppressor cell activity, specifically those with  $H_2$ -receptors.

INDO (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3 acetic acid) is a prostaglandin synthetase inhibitor. Prostaglandins (PG) inhibit immune response. They are produced endogenously in culture containing PHA (T cell mitogen) by suppressor cells and inhibit the mitogenic stimulation of T cells but not B cells. Adding INDO to a culture stimulated by T cell mitogen will block the inhibitory effects of PG on suppressor cells. Therefore, an increase in DNA synthetic activity of cells after INDO treatment is an indication of suppressor cell function.

The following experiments were designed to quantitate the suppressor T cell function using Cimetidine (CIM) and Indomethacin (INDO) in a lymphocyte culture system with the following mitogens: phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed (PW). The experiments were carried out using spleen and thymus lymphocytes from control rats. A summary of the treatments follows:

Pooled spleen and thymus lymphocytes from untreated adult Fischer 344 male rats were cultured with mitogens alone, and with mitogens plus CIM or INDO. The doses of the mitogens were PHA 2, 4, and 6%; PW 1% and Con A 0.5%. There were 2 concentrations of CIM (0.125  $\mu\text{g/ml}$  and 0.25  $\mu\text{g/ml}$ ), and 2 concentrations of INDO (1.0  $\mu\text{g/ml}$  and 1.5  $\mu\text{g/ml}$ ). Cells were preincubated with either CIM or INDO for 0, 1 or 2 hours prior to exposure to the mitogens. The 72 hr incubation times started with the addition of the cells to the mitogens. All other methods have been previously described.

The results of the quantitation of suppressor T cell activity in spleen and thymus lymphocytes are shown in Table 4 and Figures 4-6. Statistically significant enhancement in mitogen stimulation (using PHA, Con A and PW) was found when spleen lymphocytes were pre-incubated one hour using CIM 0.125  $\mu\text{g/ml}$  or INDO 1.0  $\mu\text{g/ml}$ . Dose response relationships were found using PHA 2%, 4% and 6% with and without CIM and INDO.



TABLE 4: Measurement of Suppressor Cell Activity in Spleen Lymphocytes using Mitogen Activation in the Presence of Cim or Indo at Selected Concentrations

Mitogens	Mitogen only dpm/10 <sup>5</sup> cells		Cim .125 ug/ml		Cim .25 ug/ml		Indo 1.0 ug/ml		Indo 1.5 ug/ml	
	dpm ± SD	%	dpm ± SD	%	dpm ± SD	%	dpm ± SD	%	dpm ± SD	%
DPM ± SD* and % Increase in Spleen Lymphocytes After No Preincubation										
Control	1059 ± 136		1536 ± 185	45	1626 ± 110	54	1227 ± 52	16	1474 ± 197	39
PHA 2%	8689 ± 636		7618 ± 641	0	6623 ± 444	0	7127 ± 1733	0	7890 ± 1970	0
4%	13736 ± 1203		9778 ± 875	0	8840 ± 671	0	12292 ± 2170	0	10459 ± 1773	0
6%	15675 ± 1658		11109 ± 1317	0	11329 ± 1090	0	10805 ± 843	0	10175 ± 870	0
PWM1%	4619 ± 513		6519 ± 1127	41	6908 ± 397	50	6224 ± 342	35	6758 ± 845	46
ConA5%	17746 ± 2399		10830 ± 282	0	14247 ± 718	0	12698 ± 546	0	10486 ± 738	0
DPM and % Increase in Spleen Lymphocytes After 1 hr Preincubation										
Control	1059 ± 136		1291 ± 108	22	1034 ± 93	0	934 ± 71	0	861 ± 115	0
PHA 2%	8689 ± 636		10650 ± 374	23	9535 ± 597	10	10071 ± 450	16	8609 ± 460	0
4%	13736 ± 1203		13812 ± 286	0	10208 ± 1531	0	15044 ± 1193	10	14200 ± 240	3
6%	15675 ± 1658		17020 ± 425	9	16614 ± 677	6	18863 ± 1465	20	18501 ± 2484	18
PWM1%	4619 ± 513		5352 ± 567	16	4560 ± 17	0	6215 ± 462	35	5357 ± 345	16
ConA5%	17746 ± 2399		19061 ± 337	1	20560 ± 1770	16	21481 ± 1946	21	19374 ± 2054	9
DPM and % Increase in Spleen Lymphocytes After 2 hr Preincubation										
Control	1059 ± 136		1200 ± 45	13	1491 ± 117	41	1294 ± 252	22	1020 ± 182	0
PHA 2%	8689 ± 636		8863 ± 668	2	8990 ± 239	3	8656 ± 606	0	7416 ± 464	0
4%	13736 ± 1203		10960 ± 389	0	10170 ± 771	0	9616 ± 863	0	9818 ± 798	0
6%	15675 ± 1658		14830 ± 1426	0	11723 ± 531	0	14387 ± 603	0	15509 ± 1017	0
PWM1%	4619 ± 513		6314 ± 835	37	5237 ± 471	13	5359 ± 726	16	3965 ± 259	0
ConA5%	17746 ± 2399		12449 ± 1045	0	12658 ± 873	0	15065 ± 489	0	12476 ± 813	0

\*Each experiment consists of 4 replicates from pooled lymphocytes of 2 rats per group.

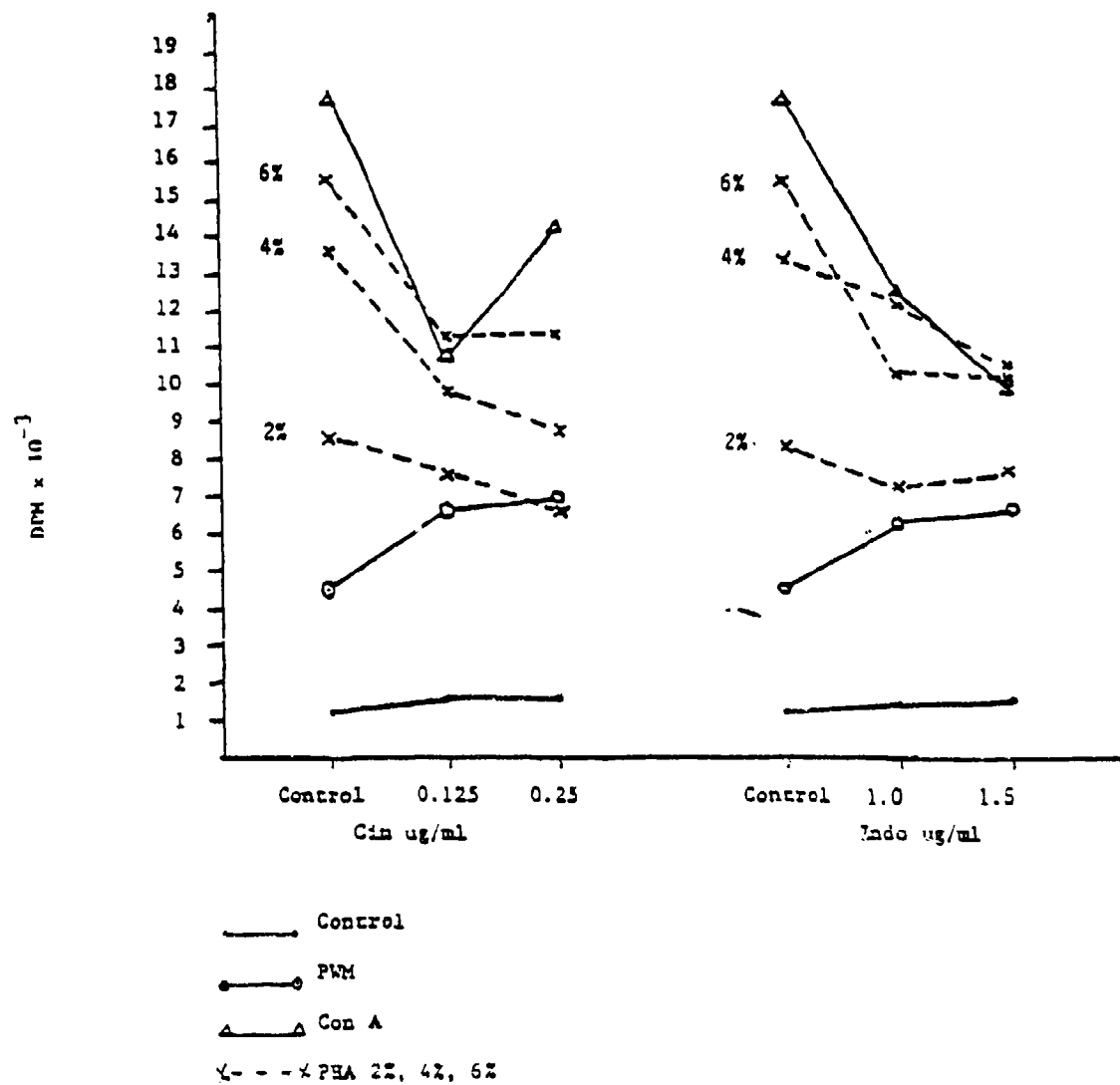


FIGURE 4: SPLEEN LYMPHOCYTES  
No Preincubation

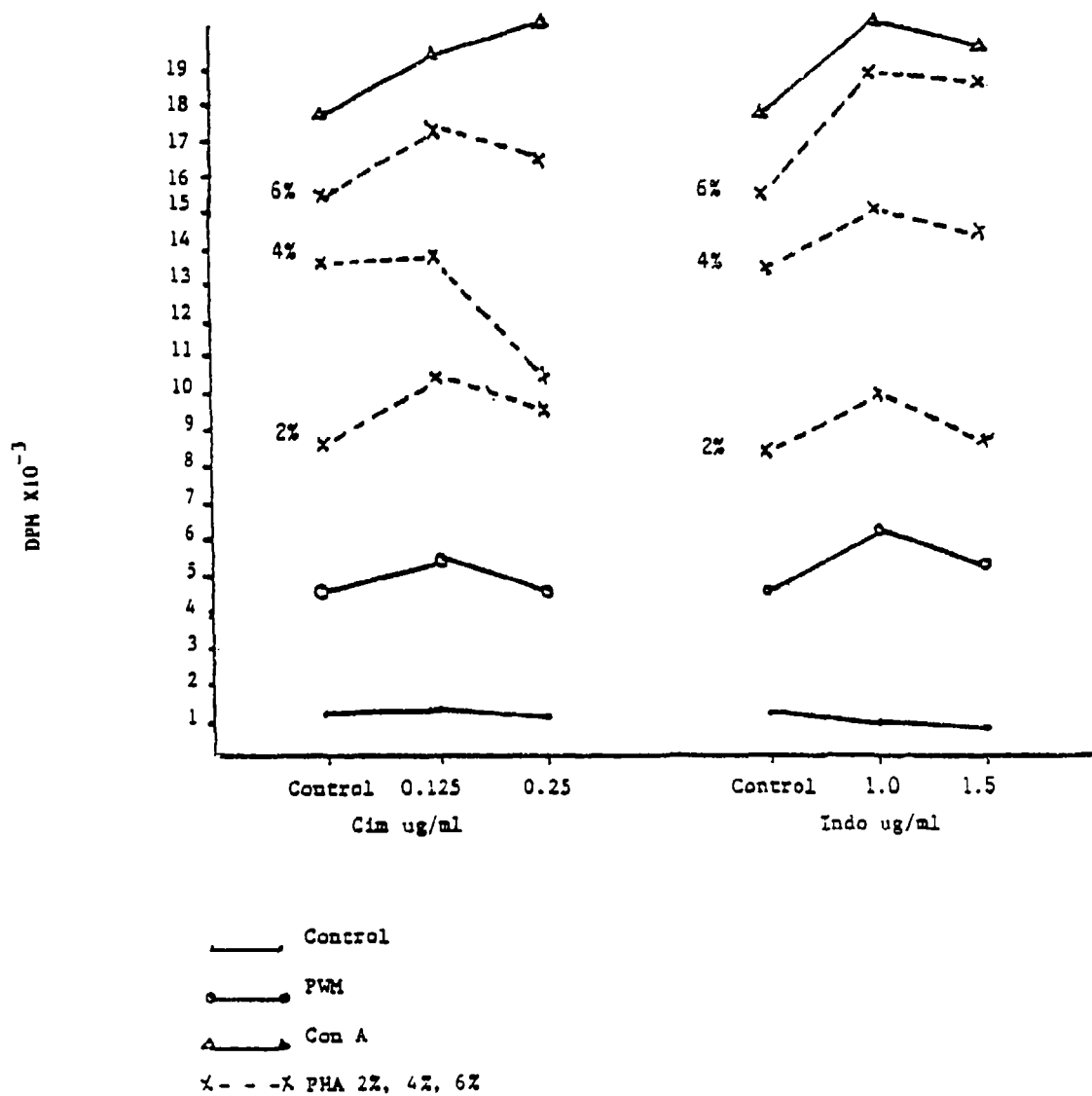


FIGURE 5: SPLEEN LYMPHOCYTES  
1 Hr. Preincubation

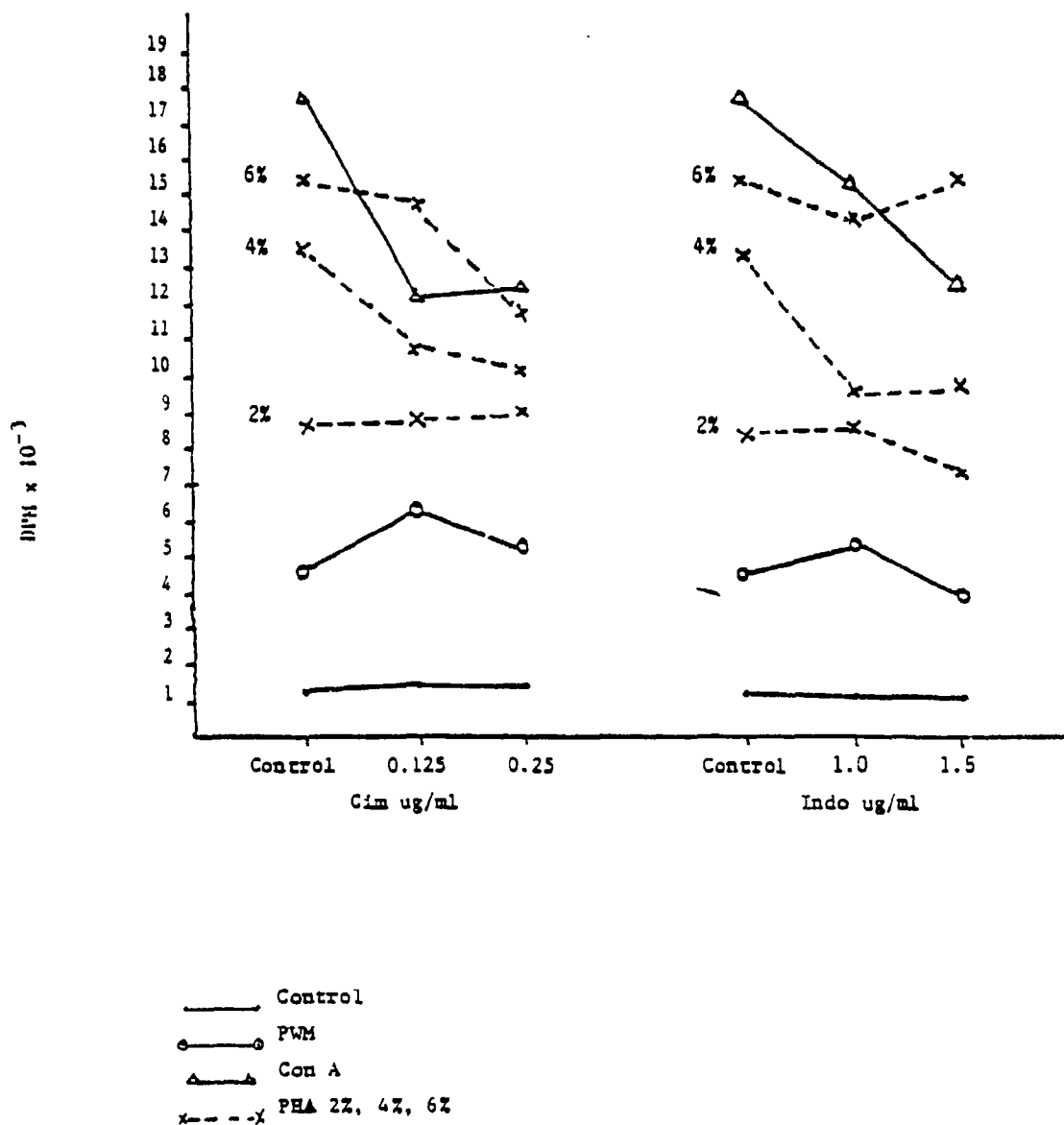


FIGURE 6 :      SPLEEN LYMPHOCYTES  
2 Hr. Preincubation

Results for thymocytes (Table 5 and Figures 7 and 8) show that the greatest enhancement was found when 0.25 µg/ml CIM or 1 µg/ml INDO were used with no preincubation. No difference was found when cells were preincubated with CIM or INDO for 2 hours.

#### DNA Repair Synthesis in Rat Spleen and Thymus Lymphocytes

DNA replication and repair synthesis in pooled spleen lymphocytes were measured to compare effects of exposure to 4-nitroquinoline oxide (4NQO) and to UV light. Table 6 shows the results on spleen lymphocytes using different concentrations of 4NQO and exposure to UV. As can be seen, more repair was found using the lower concentration of 4NQO ( $5 \times 10^{-6}M$ ) although replication was not significantly different using the two concentrations. It can also be seen that the  $10^{-5}M$  concentration of 4NQO was toxic and no DNA repair activity was observed at the three hour incubation period.

UV exposure was also used to induce unscheduled DNA repair synthesis. The UV source (shortwave UV lamp, Minerallight, UVS-11 from Ultra-violet Products, Inc., 115 volt, 60 cycles, 0.12 amps, 250 nm wavelength) is placed in position at a height allowing 1 joule/m<sup>2</sup>/sec as determined by use of a Jagger type ultraviolet meter which has been calibrated against a Yellow Springs UV meter. An uncovered petri dish containing 10 ml cell-suspension at  $2 \times 10^6$  lymphocytes per ml in phosphate buffered saline is centered below the UV source during the duration of treatment. After treatment the cells are centrifuged, resuspended in RPMI 1640 medium, counted and viability determined before use. The results show that thymus lymphocytes demonstrate no repair using 4NQO and minimum repair with UV treatment. Using rat spleen lymphocytes, DNA repair synthesis was found after exposure to 4NQO, while UV treatment at most exposure times induced higher repair. UV treatment for 20 seconds appears to induce maximum DNA repair synthesis. A summary of the results is presented in Figure 9 where it is shown that replication was greater at a 3-hour incubation period than at a 2-hour incubation period and that DNA repair was higher when cells were exposed to UV for 20 seconds.

#### Sister Chromatid Exchanges (SCE) in Rat Spleen Lymphocytes 48 Hours, 7 Days and 14 Days after Intratracheal Instillation with 3-Methylcholanthrene (MCA)

##### Methods of Rat Treatment.

Male Fischer 344 rats (Charles River Laboratories) approximately 3 months old (age estimated by body weight, which was 300 g) were

TABLE 5 : Measurement of Suppressor Cell Activity in Thymocytes Using Mitogen Activation  
in the Presence of Cim and Indo at Different Concentrations

Mitogens	Mitogen only dpm/10 <sup>5</sup> cells		Cim .125 ug/ml		Cim .25 ug/ml		Indo 1.0 ug/ml		Indo 1.5 ug/ml	
	dpm	± SD		%		%		%		%
DPM ± SD* and % Increase with No Preincubation in Thymocytes										
Control	1944	± 76	4487	± 262 131	4472	± 579 130	3680	± 286 89	3042	± 281 56
PHA 2%	8501	± 1061	11567	± 472 36	12778	± 822 50	11064	± 1071 30	10029	± 210 18
4%	11963	± 1760	18851	± 1279 56	23367	± 3494 95	18557	± 1584 55	18042	± 900 51
6%	21460	± 572	27472	± 46 28	25924	± 878 21	30753	± 17 43	25540	± 1968 19
PWM 1%	15853	± 1939	29010	± 1587 83	38680	± 5710 144	30196	± 2389 90	26178	± 2056 65
ConA 5%	38345	± 2183	50403	± 5252 31	57575	± 7844 50	56611	± 7056 48	46139	± 128 20
DPM and % Increase After 2 hr Preincubation with Cim and Indo in Thymocytes										
Control	1944	± 76	5198	± 575 167	5034	± 506 159	4697	± 156 142	3875	± 751 99
PHA 2%	8501	± 1061	12856	± 1428 51	15780	± 1337 86	15108	± 2384 78	10198	± 851 20
4%	11963	± 1760	17963	± 1861 50	22269	± 5670 86	20944	± 3324 75	18054	± 653 51
6%	21460	± 572	25487	± 2355 19	24831	± 2095 16	29169	± 3756 36	22205	± 1490 3
PWM 1%	15853	± 1939	30357	± 2244 91	35462	± 4220 124	35672	± 6594 125	25988	± 584 64
ConA 5%	38345	± 2183	53325	± 5208 39	57564	± 8677 50	55533	± 10355 45	49555	± 1689 29

\*Each experiment consists of 4 replicates from pooled lymphocytes of 2 rats per group.

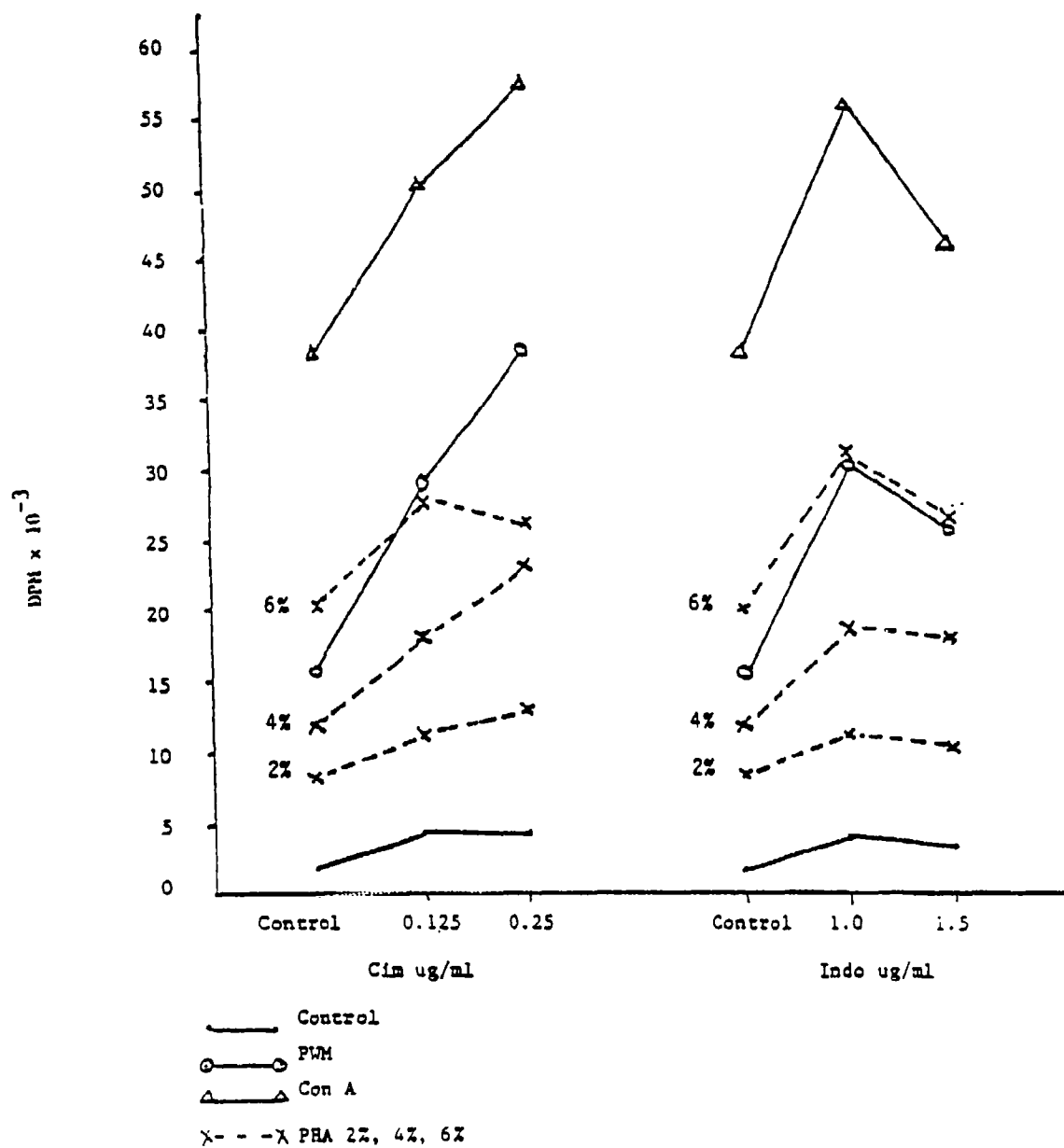


FIGURE 7 : THYMOCYTES  
No Preincubation

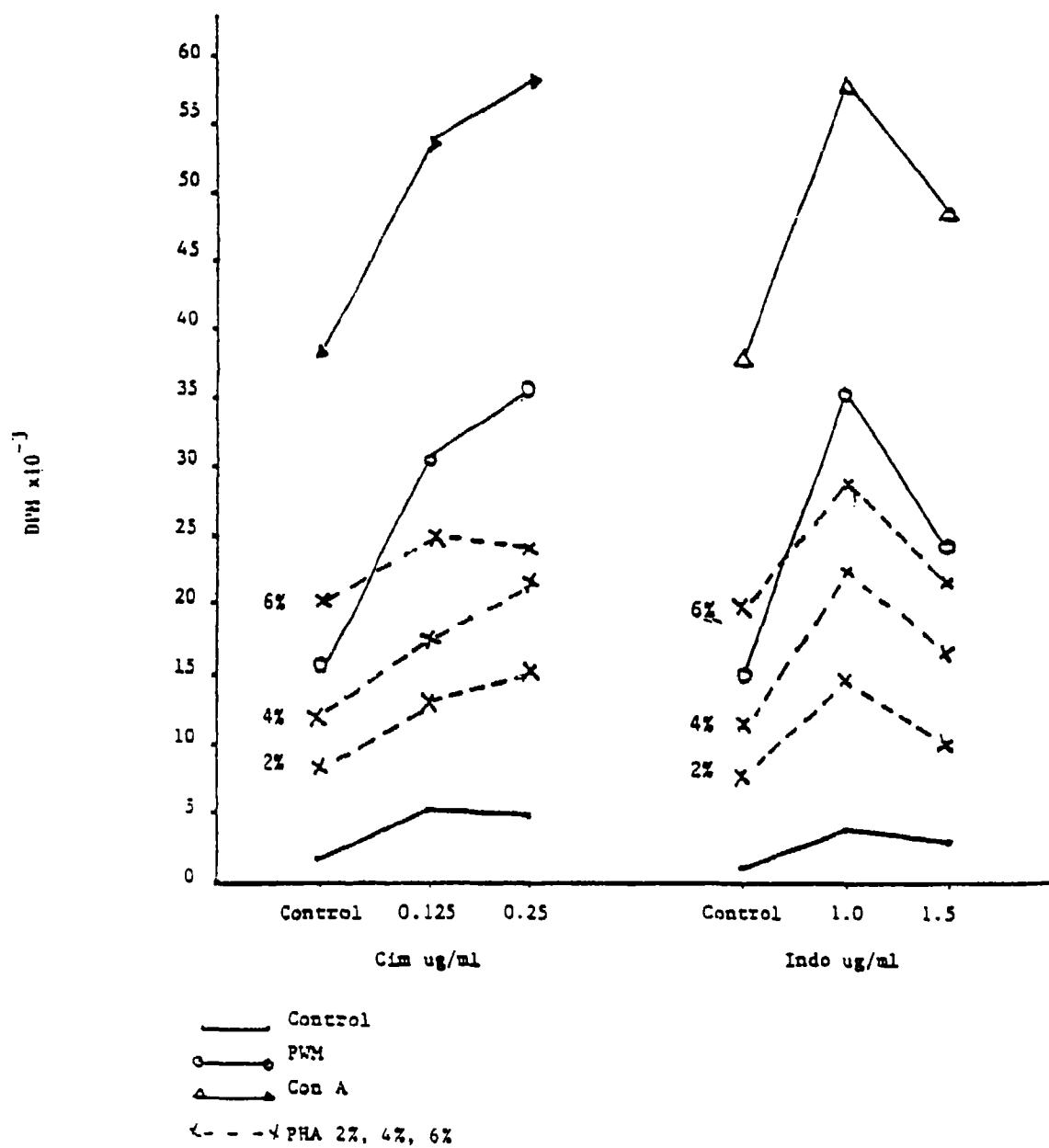


FIGURE 8 : THYMOCYTES  
2 Hr. Preincubation



TABLE 6 : DNA REPLICATION AND REPAIR SYNTHESIS IN RAT SPLEEN CELLS  
EFFECTS OF INCUBATION TIME AND EXPOSURE TO 4NQO AND UV EXPOSURE

<u>Treatment</u>	<u>Control Replication</u>	<u>HU</u>	<u>Treated Cells</u>	<u>Treated + HU</u>	<u>DNA Repair</u>
Incubation Time: 2 Hours					
DPM $\pm$ S.D./2 x 10 <sup>5</sup> cells					
4NQO					
1 x 10 <sup>-5</sup> M	1331 $\pm$ 266	140 $\pm$ 53	900 $\pm$ 118	293 $\pm$ 18	153 $\pm$ 40
5 x 10 <sup>-6</sup> M			1027 $\pm$ 27	269 $\pm$ 43	129 $\pm$ 48
*UV 10 sec.	1250 $\pm$ 41	136 $\pm$ 70	1545 $\pm$ 83	427 $\pm$ 73	291 $\pm$ 72
15 sec.			1672 $\pm$ 62	474 $\pm$ 63	338 $\pm$ 67
20 sec.			1636 $\pm$ 56	424 $\pm$ 14	288 $\pm$ 50
Incubation Time: 3 Hours					
4NQO					
1 x 10 <sup>-5</sup> M	2173 $\pm$ 112	106 $\pm$ 33	660 $\pm$ 68	165 $\pm$ 14	59 $\pm$ 25
5 x 10 <sup>-6</sup> M			1122 $\pm$ 124	353 $\pm$ 75	247 $\pm$ 58
UV 10 sec	1951 $\pm$ 128	137 $\pm$ 22	1839 $\pm$ 145	425 $\pm$ 16	288 $\pm$ 19
15 sec			2099 $\pm$ 75	529 $\pm$ 56	392 $\pm$ 43
20 sec			1951 $\pm$ 52	685 $\pm$ 103	548 $\pm$ 74
UV is delivered at 1.38 x 10 <sup>8</sup> ergs/sec.					

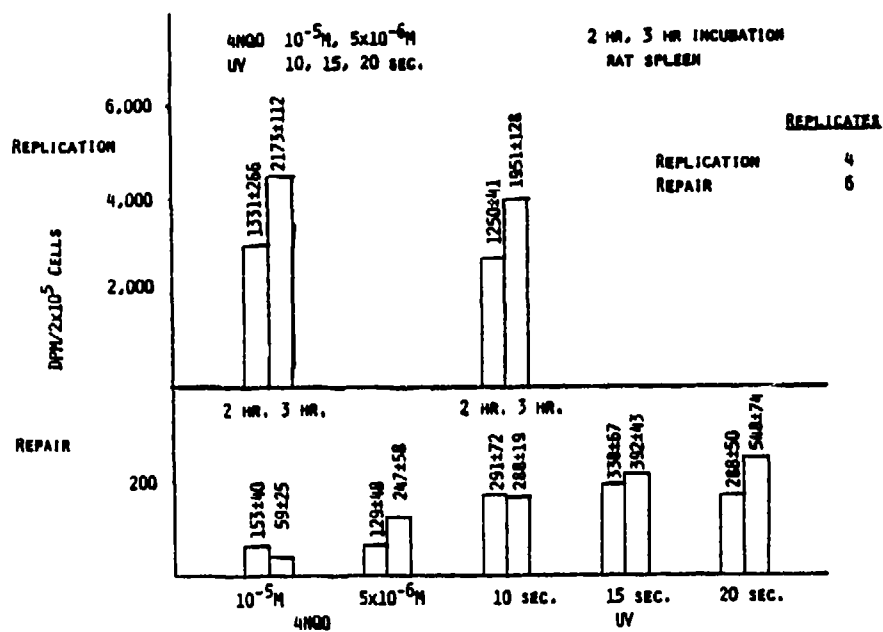


FIGURE 9: DNA REPAIR IN CONTROL RATS

used in this set of experiments. Animals were anesthetized with methoxyflurane and inoculated intratracheally with a suspension of 3-methylcholanthrene (MCA) in 0.2% gelatin in 0.9% NaCl. The anesthesia was maintained during surgery by supplying methoxyflurane to the rats' noses in a small plastic funnel. The surgery consisted of making a small slit in the neck and injecting the MCA suspension between the rings of the trachea; the wound was closed with one or two clips. The MCA suspension was prepared by sonication of the MCA crystals in gel saline for about an hour. Microscopic examination indicated that most of the MCA was dispersed in particles of 1  $\mu$ m in diameter. Four groups of controls are included: untreated, sham surgery, vehicle and anesthesia only. Seven days and 14 days after MCA treatment the rats were anesthetized with sodium pentobarbital and sacrificed by desanguination via heart puncture. Using aseptic techniques the spleens were removed and placed in test tubes containing sterile Roswell Park Memorial Institute (RPMI) tissue culture medium 1640.

Lymphocytes from rat spleens were isolated, counted and suspended in RPMI tissue culture medium containing 20% fetal calf serum (FCS), 4% Phytohemagglutinin (PHA) and 0.5% BrdU. Cultures were kept in a 5% CO<sub>2</sub> incubator at 37°C for 64 hours. One hour before harvest colcemid solution was added to each culture. Cell cultures were centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and 0.075 M potassium chloride (KCl) solution was added to each culture tube. After 10 minutes, culture tubes were centrifuged and the supernatant was discarded. Cells were then fixed with methanol-glacial acetic acid mixture (75% methanol, 25% glacial acetic acid V/V). Cells were plated on microscope slides and left to dry for 24 hours before staining.

The dried slide preparations were then stained with Hoechst stain for 10 minutes, then exposed to fluorescent lights for 3 hours, and stained with Giemsa stain. Slides were cover slipped and then examined microscopically. The criteria for microscopic examination included the total number of metaphases, first and second divisions, the number of chromosomes per metaphase and the quality of the differential staining.

SCEs were counted only in metaphases with at least 38 chromosomes. The number of SCEs were counted per metaphase and frequency per metaphase calculated. The mean and standard deviation were calculated for the frequency of SCEs per metaphase and results are presented in Table 7 and Figures 10 and 11.

TABLE 7 Frequency of Sister Chromatid Exchanges in Spleen Lymphocytes from Rats Intratracheally treated with 3-Methylcholanthrene (MCA) and Control Rats

Rat Treatment	Time After Treatment	No. of Metaphases	$\bar{x}$ number of Chromosomes per metaphase	$\bar{x}$ number of SCE per metaphase	$\bar{x}$ frequency of SCE	S.D.
Untreated Controls	48 hrs.	24	40.2	6.2	0.16 (0.12)	0.03 (0.05)
Vehicle Controls	48 hrs.	31	41.1	6.1	0.16 (0.20)	0.04 (0.05)
MCA treated	48 hrs.	26	40.1	8.1	0.20 (0.25)	0.07 (0.06)
Untreated Controls	7 days	28	39.2	7.5	0.19 (0.18)	0.04 (0.06)
Vehicle Controls	7 days	30	41.0	7.1	0.17 (0.19)	0.03 (0.05)
MCA treated	7 days	31	40.4	8.7	0.22 (0.28)	0.07 (0.07)
Untreated Controls	14 days	31	40.9	7.7	0.19 (0.19)	0.04 (0.03)
Vehicle Controls	14 days	32	40.9	8.1	0.20 (0.21)	0.04 (0.08)
MCA treated	14 days	32	40.8	7.9	0.19 (0.16)	0.04 (0.08)

( ) Figures if photography is used for each metaphase before counting SCE's

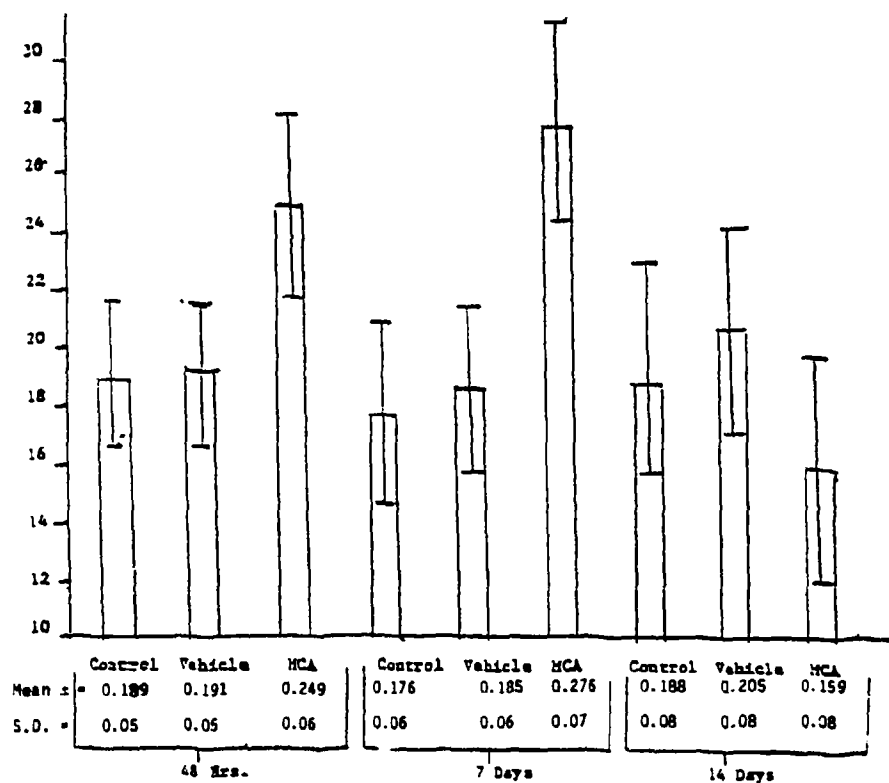


FIGURE 10: Figures represent frequency of sister chromatid exchanges (SCEs) to the total number of chromosomes in the respective chromosomal spread. The results in Figure 10 were taken from photographs of these spreads.

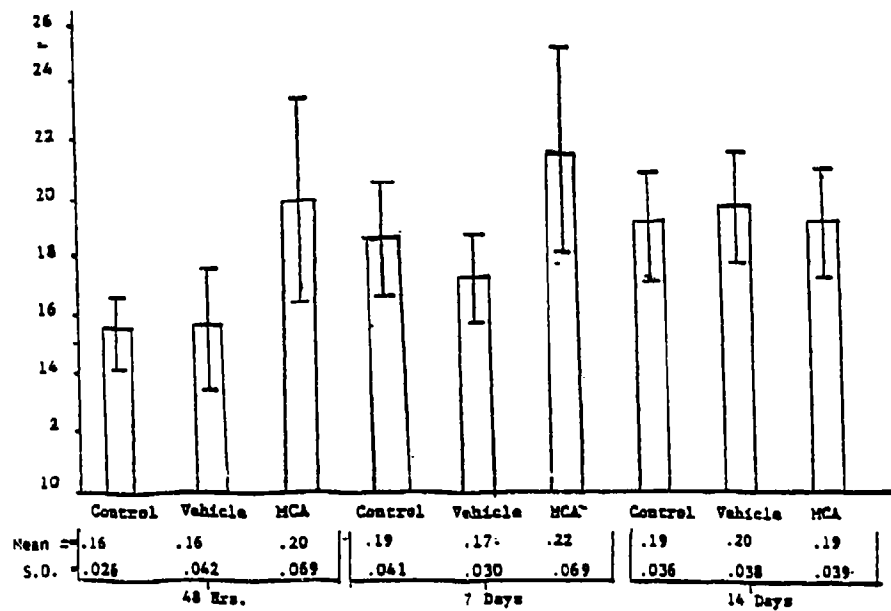


FIGURE 11: Figures represent frequency of sister chromatid exchanges (SCEs) to the total number of chromosomes in the respective chromosomal spread. The results in Figure 11 were taken from microscopic examination of these spreads.

As can be seen, the mean frequency of SCE per metaphase ranged from 0.16 to 0.22 using microscopic examination and 0.16 to 0.28 when counting was done using photographs. The highest standard deviation using microscopic examination was 0.07 and it was 0.08 using photographs.

The SCE frequency in spleen lymphocytes from rats 48 hours after treatment was higher in the MCA treated animals when compared with the untreated and the vehicle controls. Seven days after intratracheal instillation treatment the frequency of SCEs was also higher in the MCA treated rats compared with the control groups. Fourteen days after treatment there was no difference between the three groups. Similar results were found in both microscopic examinations and when SCE counts were done using photographs.

#### STUDIES OF IMMUNOCHEMISTRY IN RATS TREATED WITH MCA

The first attempt to induce lung tumors in Fischer 344 rats by the intratracheal (i.t.) inoculation of a suspension of 3-methylcholanthrene (MCA) in gel-saline was not successful (this work was done at the THRU). The negative result was attributed to the small particle size of the MCA (ca. 1  $\mu$ m). A second attempt was initiated in late January 1982 using a preparation of MCA having particles in the range of 100  $\mu$ m. This procedure was adopted after consultation with other investigators who have induced tumors in mice with MCA suspensions. The protocols and dosages are essentially the same as for the first attempt, except for the MCA particle size. Formation of the first tumors was observed in April 1982. The experiment was scheduled to run until October. Successful induction of lung tumors will provide the basis for initiation of a larger experiment involving shipment of tissues to UCI and cytological analysis of lungs of the treated rats as originally planned.

At UCI experiments have been planned for the current contract year. The results will provide basic information on the effects of intratracheal MCA and will indicate the nature of changes which may occur during the tumor initiation studies.

Rats were inoculated with 1 mg of MCA in 0.1 ml of gel-saline. Sample groups of 4 rats each (treated, sham, and untreated) were sacrificed at 2 days and at 1, 2, 3, 4, 5, and 6 weeks following MCA. The experimental schedules are shown in Table 8. Factors measured and results are summarized below.

TABLE 8: EXPERIMENT I

Six week follow-up -- Single Treatment Experiment of Rats

Carcinogen: 3-Methylcholanthrene (3MCA) large particles

Dose: 1 mg per rat

Method of Treatment: Intratracheal inoculation

Groups of Animals: Untreated, vehicle control and 3MCA

PROTOCOL

<u>Date</u>	<u>Treatment</u>	<u>Period Post Treatment</u>	<u>Total # Animals</u>	<u>Groups of Animals</u>		
				<u>Untreated</u>	<u>Vehicle</u>	<u>3MCA (1 mg/rat)</u>
1/1/82	Intratracheal Inoculation		72	24	24	24
1/3/82	"		12	4	4	4
2/5/82	Sacrifice	49 hr	12	4	4	4
2/9/82	"	1 wk	12	4	4	4
2/16/82	"	2 wk	12	4	4	4
2/23/82	"	3 wk	12	4	4	4
3/2/82	"	4 wk	12	4	4	4
3/9/82	"	5 wk	12	4	4	4
3/16/82	"	6 wk	12	4	4	4



### In Vitro Mitogen Stimulation

The effect of three mitogens on spleen and thymus lymphocyte stimulation was done and summary results are shown in Table 9. The mitogens used are phytohemagglutinin (PHA) leucoagglutinin (Leuco) and Concanavalin A (Con A). The methods used are the same as those described earlier in this report. As can be seen spleen lymphocytes show elevated response to mitogens in the 3-MCA treated groups after 48 hours. This was particularly true in the case of PHA using 3 concentrations (2%, 4%, and 6%). No significant differences were found between the mitogen activation in spleen lymphocytes from vehicle treated rats and 3-MCA treated animals in weeks after treatment until the fourth week, when a depression was observed particularly when Leuco and Con A were used (Figure 12). In Figure 12 the data are expressed as % of DPM in the 3-MCA treated of that of the vehicle. Figure 12 also shows that by week 6 there was a depression of mitogen stimulation using all three doses of PHA. It is of interest that tumors are expected to be observed at 6-8 weeks after 3-MCA treatment.

Table 10 and Figure 13 show the results of mitogen activation in thymocytes from animals intratracheally treated with 3-MCA. Again 48 hours after treatment, there was an increase in mitogen stimulation using Con A and PHA which was not observed using Leuco. It was also clear that PHA stimulation at 6 weeks after treatment was lower in 3-MCA treated animals than in the vehicle treated rats.

### DNA Repair Synthesis

The methods have been described earlier. The results are presented in Tables 11 and 12. DNA replication at 48 hours after 3-MCA treatment was significantly reduced when compared with the vehicle control. The proportion of replication in 3-MCA treated animals in relation to vehicle treated rats increased and showed consistently higher replication in 3-MCA treated animals for all six weeks after treatment. DNA repair results are shown in Table 12. They show that after 3-MCA treatment, DNA repair was increased in the treated animals compared to vehicle animals.

### Sister Chromatid Exchange (SCE) in Spleen Lymphocytes

Methods used for SCE preparation were described earlier. The results presented in Table 13 show mean SCEs per chromosome in vehicle treated and 3-MCA treated animals. As can be seen, there was no significant difference in the frequency of SCEs 48 hours and

TABLE 9: Mitogen Stimulation in Rat Spleen Lymphocytes After Intratracheal Treatment with 3-MCA ( $\bar{x} \pm S.D.$ )

A. Vehicle Treated Animals

Mitogen	48 hours	Time Posttreatment				
		1 week	2 weeks	3 weeks	4 weeks	5 weeks
Control	756 $\pm$ 46	5,808 $\pm$ 1,012	1,601 $\pm$ 197	37,463 $\pm$ 3,608	1,165 $\pm$ 153	511 $\pm$ 30
Leuco	7,731 $\pm$ 540	22,620 $\pm$ 2,406	16,765 $\pm$ 1,675	1,805 $\pm$ 147	21,089 $\pm$ 1,645	13,655 $\pm$ 1,821
Con A	6,350 $\pm$ 616	60,555 $\pm$ 6,555	52,061 $\pm$ 9,371	27,367 $\pm$ 3,731	25,789 $\pm$ 3,838	19,752 $\pm$ 1,650
PHA 28	4,729 $\pm$ 1,178	28,808 $\pm$ 5,625	11,375 $\pm$ 1,377	13,320 $\pm$ 790	12,927 $\pm$ 1,433	5,343 $\pm$ 375
PHA 48	6,533 $\pm$ 381	39,367 $\pm$ 3,830	15,464 $\pm$ 1,628	18,605 $\pm$ 3,583	18,312 $\pm$ 3,846	7,627 $\pm$ 633
PHA 68	9,144 $\pm$ 682	46,238 $\pm$ 1,511	12,354 $\pm$ 1,206	30,356 $\pm$ 5,531	19,894 $\pm$ 2,519	7,269 $\pm$ 1,215

B. 3-MCA (1.0 mg) Treated Animals

Control	562 $\pm$ 17	2,579 $\pm$ 360	2,136 $\pm$ 201	1,852 $\pm$ 203	1,384 $\pm$ 110	610 $\pm$ 138
Leuco	16,083 $\pm$ 905	21,905 $\pm$ 1,803	22,618 $\pm$ 3,490	21,451 $\pm$ 4,341	13,359 $\pm$ 1,480	21,631 $\pm$ 1,974
Con A	70,995 $\pm$ 1,176	134,309 $\pm$ 16,365	79,415 $\pm$ 4,294	48,209 $\pm$ 5,918	23,920 $\pm$ 1,241	29,536 $\pm$ 222
PHA 28	19,572 $\pm$ 2,352	26,068 $\pm$ 3,178	12,580 $\pm$ 950	19,441 $\pm$ 2,627	13,585 $\pm$ 1,758	7,661 $\pm$ 747
PHA 48	32,785 $\pm$ 335	18,951 $\pm$ 3,333	17,502 $\pm$ 2,477	23,429 $\pm$ 3,775	16,338 $\pm$ 761	8,700 $\pm$ 592
PHA 68	33,447 $\pm$ 5,286	38,912 $\pm$ 2,808	15,093 $\pm$ 3,722	32,972 $\pm$ 900	16,020 $\pm$ 1,657	8,354 $\pm$ 1,113

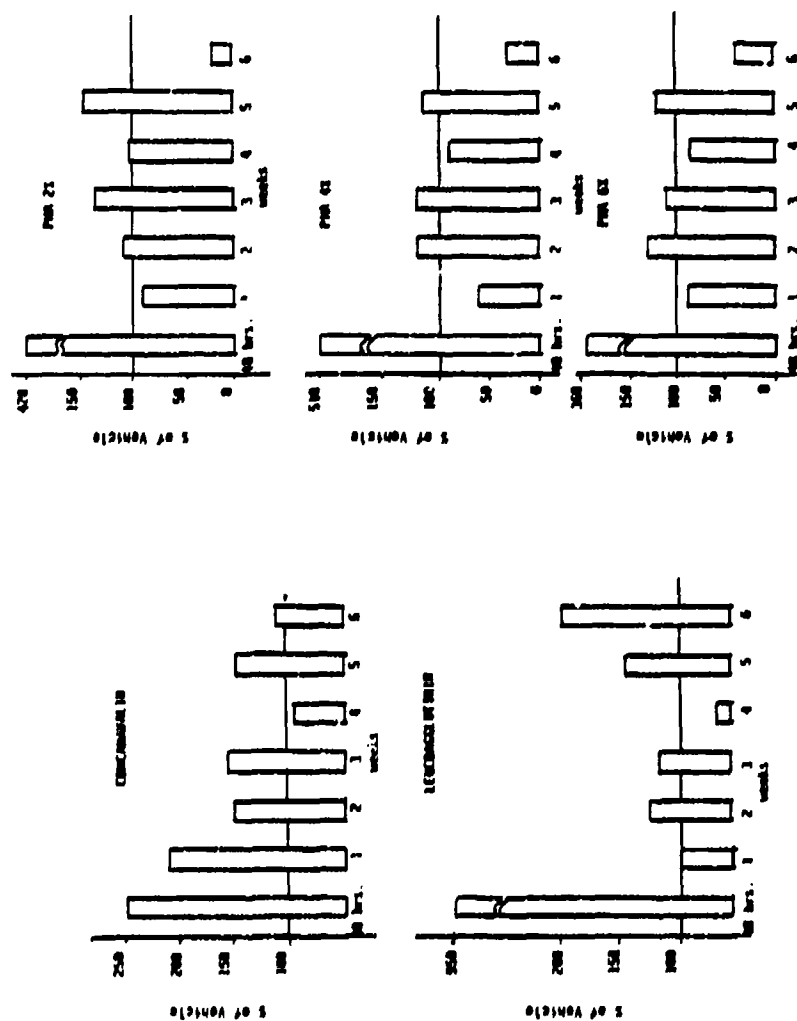


FIGURE 12: NUMBER OF SPLIC LYMOPHYTES FROM ANIMALS IMMUNIZED WITH 3 x 10<sup>6</sup> CELLS OF TUBERCLE BACILLI (RESULTS ARE EXPRESSED AS S OF VEHICLE CONCENTRATIONS)

TABLE 10: Mitogen Stimulation in Rat Thymus Lymphocytes After Intratracheal Treatment with 3-MCA (dpm  $\pm$  S.D.)

A. Vehicle Treated Animals

Mitogen	Time Posttreatment						
	48 hours	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
Control	1,034 ± 271	1,353 ± 319	1,396 ± 361	1,720 ± 303	1,173 ± 288	489 ± 55	383 ± 100
Leuco	10,707 ± 687	6,596 ± 392	14,431 ± 2,037	18,803 ± 1,428	7,266 ± 1,556	2,622 ± 276	34,612 ± 9,461
Con A	25,758 ± 1,022	17,934 ± 3,691	64,744 ± 3,598	47,060 ± 4,282	30,857 ± 3,000	30,046 ± 3,127	1,143 ± 241
PHA 28	3,694 ± 557	4,509 ± 825	7,110 ± 1,125	8,944 ± 822	3,300 ± 749	2,664 ± 484	2,717 ± 805
PHA 48	6,834 ± 528	10,027 ± 3,185	11,144 ± 2,456	18,623 ± 2,377	6,644 ± 1,248	6,038 ± 1,051	3,977 ± 451
PHA 68	11,758 ± 1,467	11,229 ± 1,358	12,395 ± 1,905	22,657 ± 1,977	8,955 ± 2,393	9,206 ± 955	7,298 ± 1,373

B. 3-MCA (1.0 mg) Treated Animals

Control	1,359 $\pm$ 115	669 $\pm$ 66	1,783 $\pm$ 348	2,086 $\pm$ 86	1,346 $\pm$ 169	654 $\pm$ 188	636 $\pm$ 220
Leuco	34,421 $\pm$ 5,433	3,046 $\pm$ 88	13,876 $\pm$ 2,046	22,625 $\pm$ 1,030	15,193 $\pm$ 1,363	4,343 $\pm$ 955	4,867 $\pm$ 827
Con A	6,658 $\pm$ 694	17,703 $\pm$ 1,182	42,983 $\pm$ 5,182	66,824 $\pm$ 8,472	53,023 $\pm$ 6,144	29,276 $\pm$ 709	51,627 $\pm$ 5,687
PHA 28	4,526 $\pm$ 112	2151 $\pm$ 200	9,798 $\pm$ 1,521	10,588 $\pm$ 1,667	6,431 $\pm$ 276	1,722 $\pm$ 149	870 $\pm$ 311
PHA 48	9,878 $\pm$ 533	5,985 $\pm$ 896	16,290 $\pm$ 2,672	23,509 $\pm$ 738	14,732 $\pm$ 767	6,145 $\pm$ 962	1,429 $\pm$ 32
PHA 68	12,677 $\pm$ 364	10,144 $\pm$ 4,029	15,703 $\pm$ 1,316	26,063 $\pm$ 3,660	15,977 $\pm$ 2,584	5,840 $\pm$ 996	3,419 $\pm$ 529

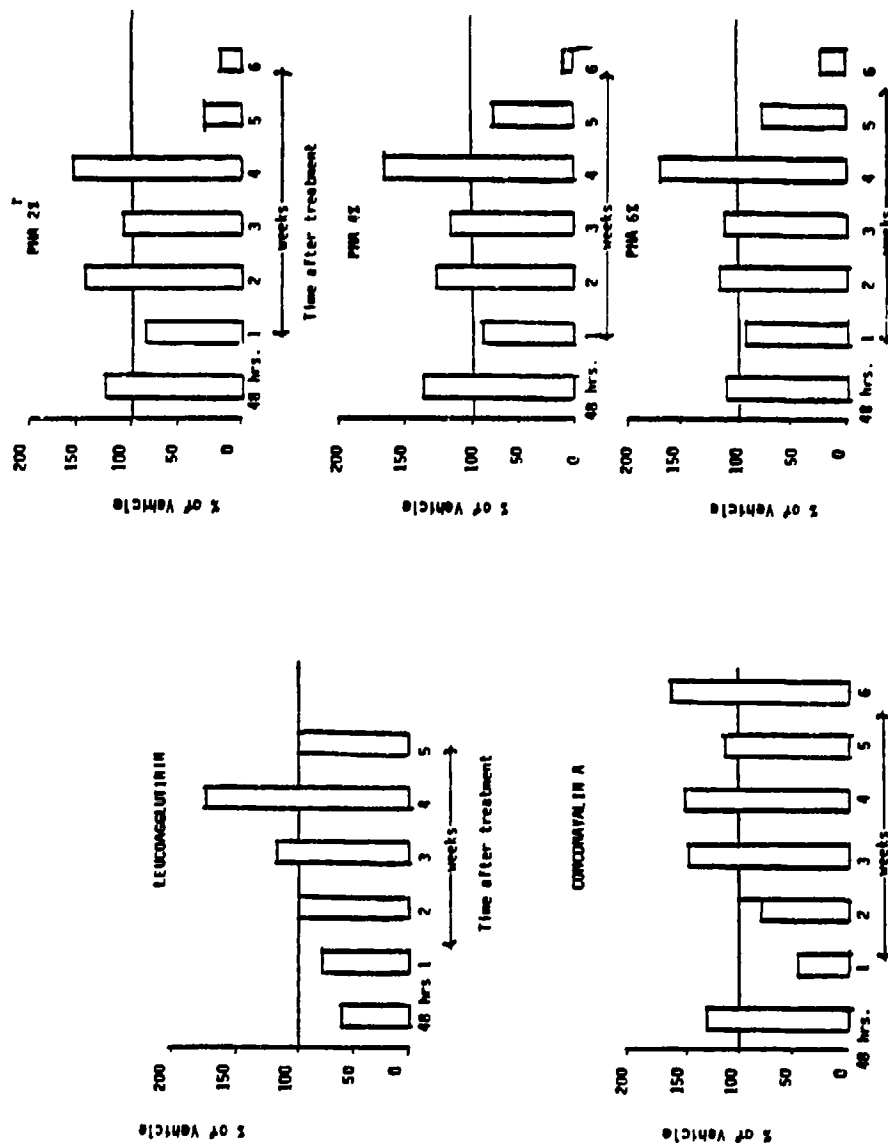


FIGURE 13: MITOGEN STIMULATION IN THYMOCYTES FROM ANIMALS INTRATHECALLY TREATED WITH 3-NCA (results are expressed as % of the vehicle control)

TABLE 11 Control Replication dpm in Spleen Lymphocytes  
From Rats Intratracheally Treated with 3-MCA  
and Vehicle Controls

Time after Exposure	Vehicle	1.0mg 3-MCA	% of Vehicle
48 hrs.	31,399	9,774	31%
1 wk.	12,496	11,457	92%
2 wks.	6,639	8,897	134%
3 wks.	3,891	6,827	175%
4 wks.	3,437	3,984	116%
5 wks.	2,120	3,181	150%
6 wks.	2,758	3,396	123%

TABLE 12 DNA Repair in Spleen Lymphocytes From Rats  
Intratracheally Treated with 3-MCA  
and Vehicle Controls

Time after Exposure	Vehicle	1.0 mg 3-MCA	% of Vehicle
48 hrs.	-	61	-
1 wk.	-	280	280%
2 wks.	194	69	36%
3 wks.	205	139	68%
4 wks.	150	567	378%
5 wks.	131	233	178%

TABLE 13: SISTER CHROMATID EXCHANGES/CHROMOSOME IN  
VEHICLE AND MCA TREATED RATS

TIME POST TREATMENT	48 Hrs.	1 Wk.	4 Wks.	6 Wks.
VEHICLE	0.18 ± 0.14	0.23 ± 0.05	0.20 ± 0.03	0.22 ± 0.10
MCA	0.19 ± 0.06	0.26 ± 0.07	0.23 ± 0.05	0.28 ± 0.06
T =	0.08	1.5	2.10	3.00
	N.S.	N.S.	P < 0.05	P < 0.01

1 week post treatment. However, there was a significant increase in the frequency of SCEs per chromosome 4 and 6 weeks after 3-MCA treatment compared with vehicle-treated animals.

#### SHORT TERM EFFECT OF INTRATRACHEAL INOCULATION OF ASBESTOS AND 3-MCA ON MITOGEN STIMULATION IN RAT SPLEEN LYMPHOCYTES

The primary aim of this experiment is to study the combined effect of asbestos and 3-MCA on the immune response measured by mitogen stimulation and also on DNA replication repair.

Rat treatment: Fischer male 344 rats 9 weeks of age were inoculated intratracheally with asbestos, 3-MCA, asbestos + 3-MCA or gel saline. Dosage was 1 mg asbestos or 3-MCA in 0.15 ml gel saline per rat. Animals were sacrificed after 48 hours. All other methods have been described previously. Mitogen doses used were as follows: PHA, 2, 4, and 6%; Leucoagglutin, 1%; Concanavalin A, 0.5%.

Results given in Figure 14 show the dose-response relationship of PHA stimulation in spleen lymphocytes in the asbestos-treated animals; however, the highest level was at 4% PHA concentration. It is seen also that 3-MCA and asbestos has the highest effect on the lymphocyte stimulation. The lowest stimulation was found in the vehicle-treated animals. In Figure 15 similar data are presented on thymocytes. As can be seen, there is a dose response relationship in the 4 treatment groups and similar to the spleen lymphocytes. The highest activity in the asbestos-treated group was found at 4% concentration PHA; 3-MCA + asbestos-treated animals exhibited the highest PHA mitogen stimulation when compared with other treatments.

These findings confirm previous experiments in which an immune response enhancement was observed 48 hours after treatment with a chemical carcinogen.



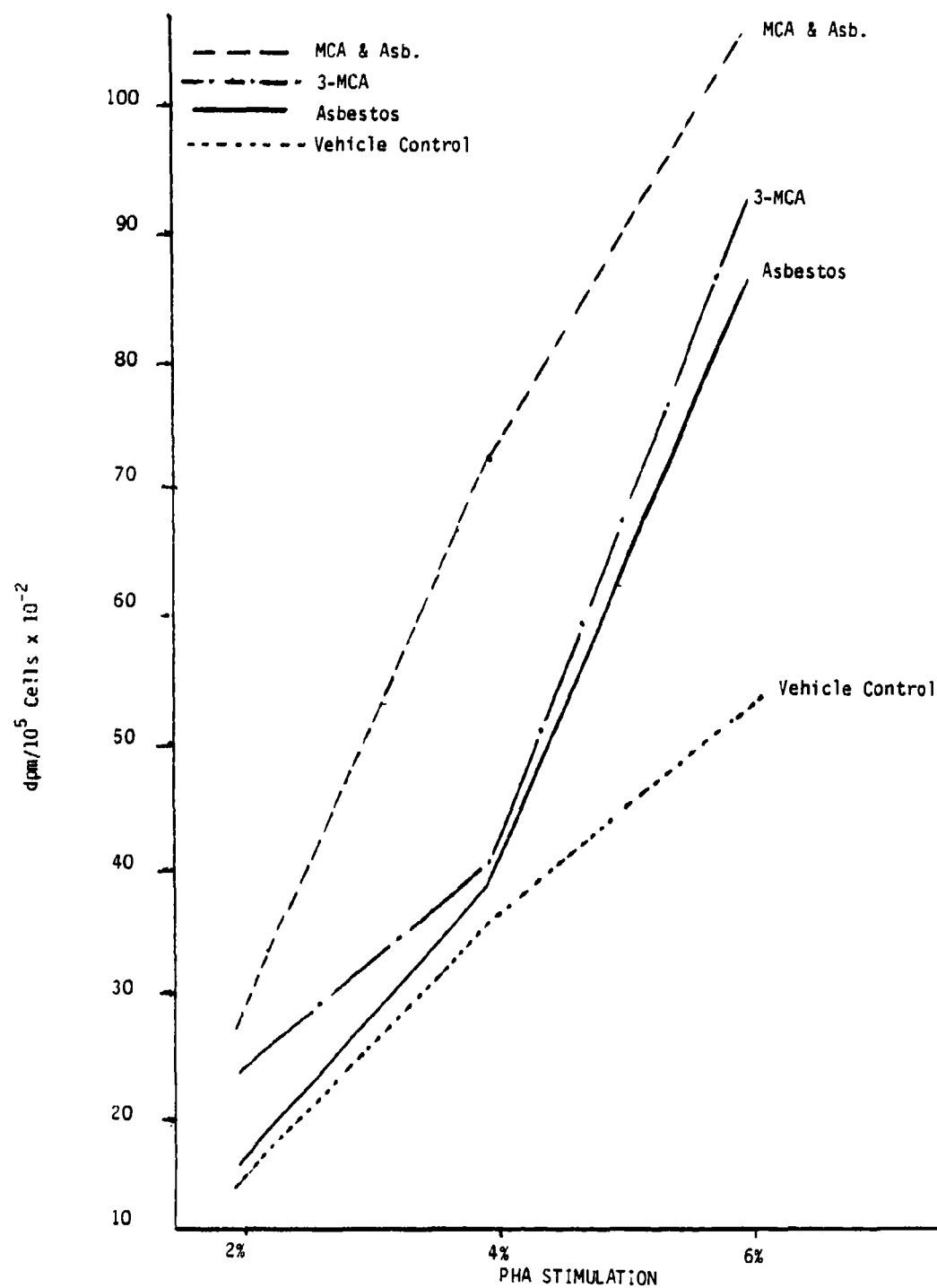


FIGURE 14: PHA MITOGEN STIMULATION IN  
THYMOCYTES 48 hrs. AFTER EXPOSURE

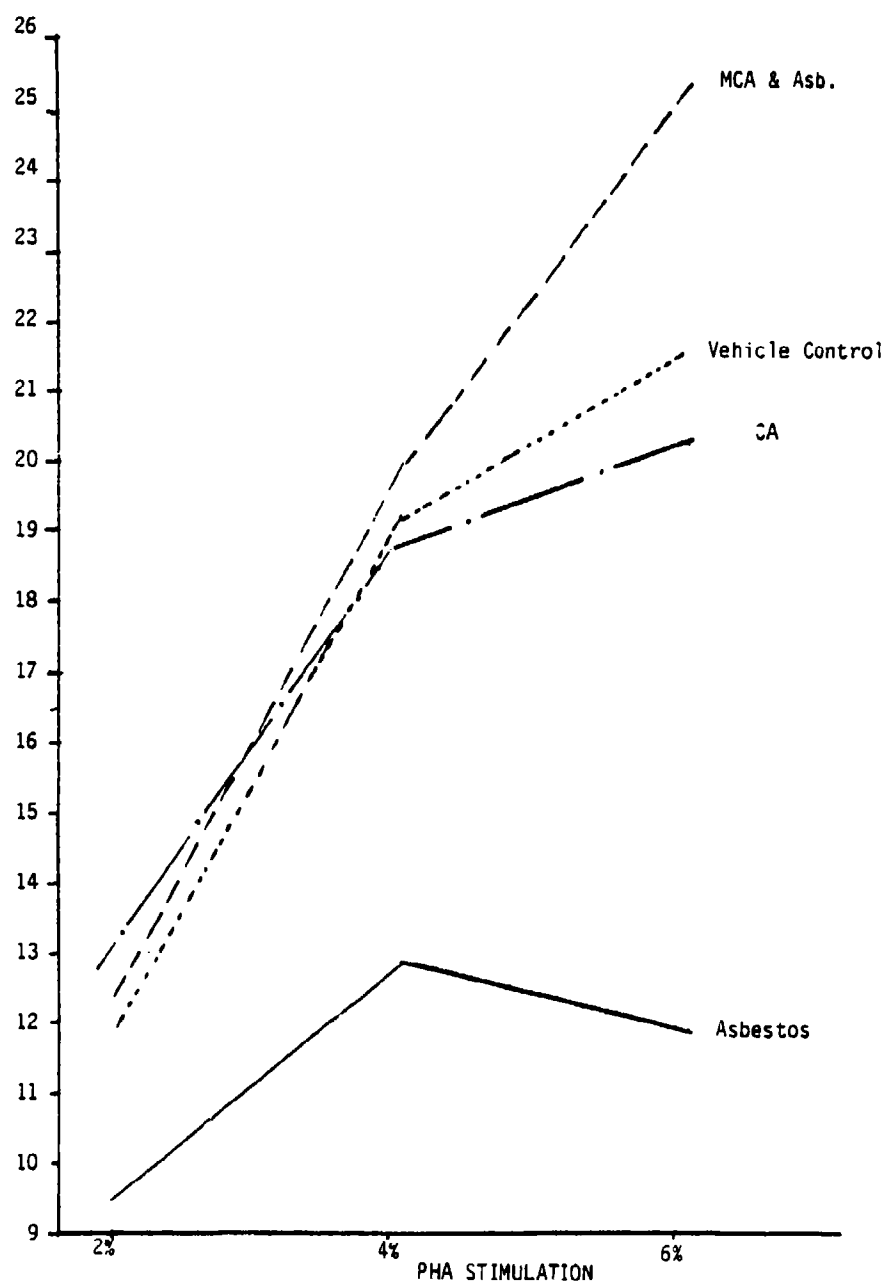


FIGURE 15: PHA MITOGEN STIMULATION IN SPLEEN LYMPHOCYTES 48 hrs. AFTER EXPOSURE

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